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IDENTIFICATION OF *Phytophthora* spp. (OOMYCOTA, PERONOSPORA) AS A CAUSAL AGENT OF STEM ROT AND DIEBACK IN FIG TREES (*Ficus carica* var. "BROWN TURKEY") OF COSTA RICA

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ABSTRACT

This research describes the morphological and molecular identification of *Phytophthora*spp. from fig (*Ficus carica* var. "brown turkey") stem and shoot samples, infected in experimental fields located in the Pacific coastal region, in the county of Turrubares, at the province of San José, Costa Rica. The study was performed in the Centro de Investigación en Biotecnología and the Laboratorio Institucional de Microscopíaof the Instituto Tecnológico de Costa Rica, by using symptomatic tree samples, from where the microorganisms of interest were isolated with V8 + Benomil (2g/l) culture medium. Growth of a cottonlike, starred and radiated micelia was observed. Through optical microscopy (OM) and Scanning Electron Microscopy (SEM) techniques, it was possible to determine the presence of spongarium and chlamydospores which are common traits of the *Phytophthora* genus. Additionally, a high quality DNA sample was obtained for the analysis through ITS markers reported for the *Phytophthora* genus. With the amplification of the IT'S region of the rDNA with the A2 and I2 primers, the presence of *Phytophthora* spp. was confirmed. The digestion of the ITS amplicon obtained with the Msp I, Rsa I and Taq I enzymes, complemented with the bioinformatic analysis of the amplified sequences allowed to confirm the presence of *P. cinnamomi* in the stem and *P. palmivora* in the shoots; being the causal agent of the fig rot.

Keywords: internal transcribed spacer region, SEM, PCR-RFLP, phylogeny, V8 selective medium.

INTRODUCTION

Fig (*Ficus carica* L.) is an important crop in different world regions (Dalkılıç*et al*., 2011). This is due mainly to the fact that its fruit presents a high nutritional value, in terms of vitamins, enzymes, flavonoids, iron, potassium and fiber that contribute to the disgestive processes, as well as its anticarcigenic and pharmaceutical properties. Apart from these traits, the ease in its propagation also make fig a versatile fruit tree for its extensive farming in a wide range of soil types, climatic conditions, and temperatures (Segreda, 2007).

In Costa Rica, figis considered a marginal species that is grown in households (Buro, 2010). However, since the year 2002, an interest was evidenced in the development of this crop as an alternative for agricultural diversification, mainly in the provinces of Cartago andSan José (Barboza,2007).Within this context, and during the crop establishment process, it is common to find issues from different origins; and therefore, it is necessary to gather knowledge related to the crop´s genetic and phenotypical characterization, propagation techniques, agronomical management, as well as the study of plagues and diseases; allowing to achieve an adequate crop management(Orozco*et al*., 2011).

The phytosanitary problems vary according to the agroclimatic conditions of each region, threatening the crop development. Research performed by Flores *et al*. (2011) and Macaya (2004),have determined that one of the main diseases that affect fig worldwide is caused by the *Phytophthora*genus, with this pathogen being the causal agent of important economic loss in certain fig producing locations. Species from *Phytophthora*genus produce a large number of diseases in many different types of plants; with the rot of the root, stem, shoots and fruit, the most common symptoms derived from this pathogen, and affecting the production yield and crop quality (Rizzo*et al*., 2002).

There are very few studies in Costa Rica and the world related to the effect of*Phytophthora* spp. in the fig crop, which limits the implementation of efficient disease control strategies in the fig producing regions. Taking this into consideration, an essential factor for the understanding of the disease dynamics is the study of the diversity in the population of the plant pathogenmicro organisms. This study has been limited since it has traditionally depended on methodologies based on microbial culture (Pallás*et al*., 2008).

Meanwhile, the use of techniques independent from the microorganism culture, based on DNA extraction methods and the execution of Polymerase Chain Reactions(PCR), have allowed considerable progress in the identification of microorganisms, providing high specificity through the design of specific primers for the markers of interest (Drenth*et al*., 2006).

The internal transcribed spacers (ITS) have been used as important markers for amplification through PCR, since they consist of highly polymorphic sequences located in ribosomal DNA (rDNA) genes; allowing the comparative analysis of highly conserved sequences through the use of their variation for phylogenetic and taxonomic studies (Pallás*et al*., 2008). Additionally, the use of restriction enzymes allows distinguishing variability at amplified sequence level, since they provide a unique genetic fingerprint for each organism. This technique is

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known as PCR-RFLP (Restriction Fragment Length Polymorphism) (Calle, 2005).

Hence, the aim of this research study was the identification to species level of the strains of *Phytophthora* causing the stem and shoot rotting in fig (*Ficus carica* var "brown turkey"), through a study of the ITS region of the rDNAand the determination of the genetic fingerprint with PCR-RFLP from samples of symptomatic trees, derived from county of Turrubares, located in the coastal Pacific region of Costa Rica, in the province of San José. This research will be the baseline for further studies on the interaction of specific pathigen strains with the local plant material, contrinuting to the fig crop management strategy in Costa Rica.

MATERIALS AND METHODS

Collecting symptomatic material, phytopathogen isolation and purification

The stem and shoot samples with apparent symptoms of *Phytophthora*spp. Infection, were gathered from fig trees presenting signs of dehydration and partial rotting, with considerably high internal humidity. Sections of 0.5 $cm^2$of symptomatic tissue were gathered and washed with tap water to remove any residues. Later, a disinfection process was performed, with 1% sodium hypochloride for 1 min, followed by three rinses with esterile distilled water (Agrios, 2005).

The disinfected tissue were placed in 9cm in diameter Petri dishes, containing 20ml of agar-V8 culture medium. After this, sections of the micellium growth presenting similar characterisctics to *Phytophthora* spp. were placed in esterile rainwater to ease the growth of fungi from the *Oomycota* phylum. Finally, sections of the micellia grown in rain water were subcultured in agar-V8 with benomil (2 ml/l). During these assays, the phytopathogen was incubated at a temperature of 27°C for a 7 day period (López & Tomás, 1999).

Morphological identification of the pathogen through Optical Microscopy (OM) and Scanning Electron Microscopy (SEM)

For the observation of the isolated microorganim through optical microscopy, sections of adult mycellium were taken and reproductive structures were analyzed; using the Nikon Eclipse e200 microscope. On the other hand, for SEM visualization, 0.5cm^2 segments of V8 agar with mycellium were processed, based on the standard method for fungi samples described by Alves *et al*. (2013) with certain modifications, consisting of a fixation stage, for 1 day at 4 °C in 2.5% glutaraldehyde and 2 % paraformaldehyde,dissolved in 0.1M phosphate buffer and a pH of 7.2, after a 2h postfixation in 2 % osmium tetroxide (OsO_4). Later, the samples were dehydrated in gradual dilutions of ethanol. The critical point drying technique was used in order to eliminate remaining liquids. Finally, the samples were observed through the TM-3000, Hitachi microscope.

Total DNA extraction, PCR-RFLP and bioinformatic analysis

The DNA extractions were performed using the DNeasy Plant Extraction Mini Kit (Qiagen®, Germany), following the manufacturer specifications, however previous to extraction, the samples were exposed to liquid nitrogen for 3 minutes along with lysis buffer, for a posterior disruption of the material through the use of a Retsch® macerator. The concentration and DNA purity was quantified through Thermo Scientific®nanoDrop Lite.

For the amplification of the ITS region, the method proposed by Drenth *et al*.(2006) was applied, using the specific primers for the regions ITS1, 5.8S and ITS2 of the rRNA of the *Phytophthora* genus: A2 (5'-ACTTTCCACGTGAACCGTTTCAA-3') and I2 (5'-GATATCAGGTCCAATTGAGATGC-3').The amplification reaction was performed in a final volume of 25 µl using DreamTaq™ Master Mix 2X (Fermentas®); 1.5 ng of the primers and 0.5 µl of the genomic DNA. The thermic profile used was of 94 °C for 2 min, (94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 1 min) x 35 cycles; and a final extension of 10 min at 72 °C. The amplified products were analyzed through 2% agarose gel electrophoresis with gelRed.

The diagnosis based in the restriction patterns was obtained as the product of the digestion of amplified sequences with three different restriction enzymes (MspI, RsaI and TaqI). To achieve this, the bioinformatic software NEBcutter®of New England BioLabs, Inc (Williams*et al*., 2007) was used, allowing to do an *in silico*PCR-RFLP*in silico*. Hence, it was necessary for each PCR product to be edited and assembled through the BioEdit (Hall, 1999) and CAP3 (Huang& Madan, 1999)programs, respectively.

The digestion patterns of each sample were ordered according to the number and the size of the base pairs of the fragments obtained from each enzyme; this way, an identification key was generated, which was compared to the *Phytophthora* species database developed by Drenth *et al*. (2006).

Finally, a phylogenetic reconstruction was created from the isolates as well as the matching accession obtained from the National Center for Biotechnology Information (NCBI), through a basic local alignment search (Blast). To perform this match, it was necessary to obtain a multiple sequence alignment using the MUSCLE tool from EMBL-EBI (Edgar, 2014). This file was analyzed with the MEGA v6.0 software (Tamura*et al*., 2013) to calculate the genetic distance between the strains based on the number of base pair substitutions between sequences and eliminating positions with missing data, through the Tamura-Neimodel (Tamura& Nei, 1993).The development of phylogenetic trees was executed through the Maximum Likelihood method with a 500-repetition bootstrap.

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RESULTS

Collecting symptomatic material, phytopathogen isolation and purification

Through the use of V8 agar with benomil (2 ml/l) as a selective culture medium for the growth of oomoycotas, it was possible to obtain the development of phytopathogens isolated from the stem and the shoots of fig trees presenting symptoms similar to those associated to infections caused by *Phytophthora* spp. (Figure-1 a and b).The isolates presented radial and starred mycellial growth and abundant aerial hyphae (Figure-1 c and d).

Figure-1. Isolation and purification of the phytopathogen (a) Stem rot in soil with excess humidity. (b) Young shoot rotting. (c and d) Microorganisms isolated from the stem and shoot samples, respectively.

Morphological identification of the pathogen through Optical Microscopy (OM) and Scanning Electron Microscopy (SEM)

Through the visualization of the microorganisms isolated from the fig stem and shoots in the optical microscope, it was possible to observe dense mycelium with long, non-segmented and branched hyphae. In regards to the microorganism isolated from the symptomatic stem, the presence of non-papilated ovoid sporangia with considerable internal content was observed (Figure 2 a and b).On the other hand, the microorganism isolated from the symptomatic fig shoot presented ellipsoid papilated sporangia and some non papilated immature sporangia, as well as coralloid hyphae (Figure-2 c).

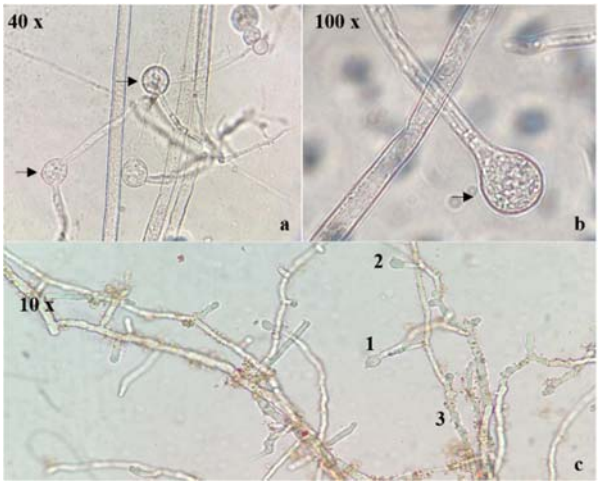

Figure-2. Morphological analysis through OM. Phytopathogen isolated from symptomatic fig stem samples: (a) Adult mycellium with presence of sporangia and chlamydospores. (b) Sporangium. Phytopathogen isolated from symptomatic fig shoot samples: (c1) Papilated sporangium. (c2) Immature non papilated sporangium. (c3) Coralloid hyphae.

The micrographs of the phytopathogen microorganism isolated from the fig tree stem, and performed through the SEM, confirmed the results gathered through OM; observing ovoid sporangia an approximate size of 33 µm. additionally, the presence of immature sporangia was also observed. Meanwhile, the samples analyzed from the phytopathogen isolated from the fig shoot, presented underdeveloped sporangia with their characteristical reproductive structure, however ellipsoid sporangia with sizes ranging the 10 µm were also observed (Figure-3).

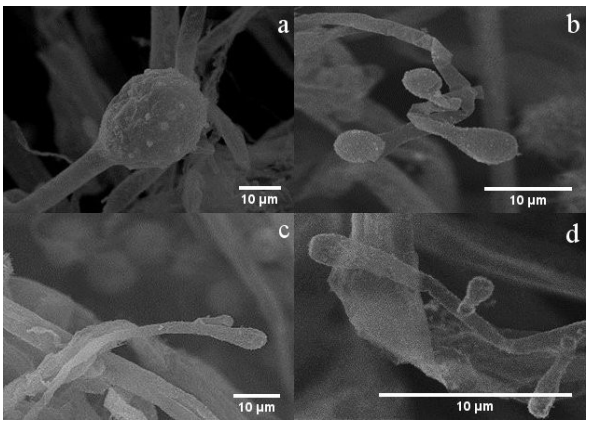

Figure-3. Micrographs of the phytopathogen microorganism isolated from the stem (a) Non papillated, ovoid shaped sporangium. (b) Development of new sporangia. (c and d) Micrographs of the phytopathogen microorganism isolated from the fig´s shoot: developing immature sporangia.

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Total DNA extraction, PCR-RFLP and bioinformatic analysis

The DNeasy Plant Mini extraction kit allowed obtaining total DNA from the P*hytophthora* isolated from symptomatic fig stems and shoots, at an average concentration of 232.45 ng/mL,with an A260/A280 mean ratio of 1.77. On the other hand, the amplification executed with specific primers for the ITS1-5,8S-ITS2 (A2 amd I2) *Phytophthora* fragment, using the total DNA isolated from symptomatic stem and shoot samples, evidenced amplified products of approximately 788 base pairs, when running the samples through electrophoresis in an agarose gel at 2% (Figure-4).

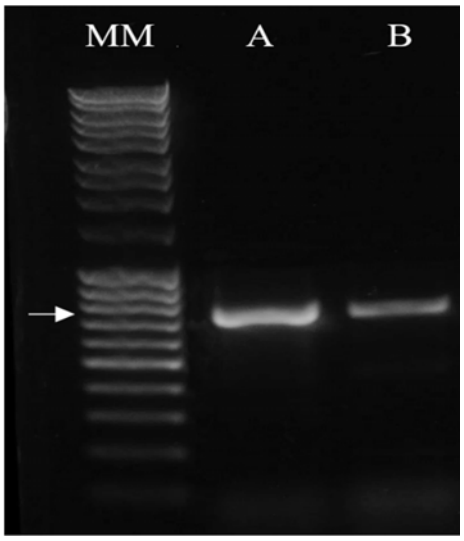

Figure-4. Bands obtained during the optimization of the PCR reaction used for the amplification of ITS1-5.8S-ITS2, from two isolates of *Phytophthora* spp. derived from the stem (A) and shoot (B) of symptomatic *F. carica*. MM: MassRuler^{TM} DNA Ladder Mix. White arrow: 800bp marker.

In regards to the digestion of the sequenced PCR with the MspI, RsaI and TaqI restriction enzymes, it was posible to determine the homologies with the restriction patterms from some of the *Phytophthora* species reported by Drenth *et al*. (2006). In Table-1, it is possible to observe the restriction patterns from the amplification of the microorganisms isolated from the symptomatic stem and shoot, where the stem isolate presented a restriction fingerprint similar to the ones for *P. cinnamoni* and *P. fragariae;* while the isolate from the shoot presented homologies with the *P. palmivora*digestion patterns.

Table-1. Digestion patterns with the MspI, RsaI and TaqI enzymes from the fig´s stem and shoot isolates (*Ficus carica* var. "brown turkey").

Microorganism isolated from	**Amplified fragment** (bp)	**Fragments generated with MspI** (bp)	**Fragments generated with RsaI** (bp)	**Fragments generated with TaqI** (bp)
Stem	797	338, 221, 146, 92	376, 170, 135, 106, 10	194, 181, 150, 90, 59, 59, 52, 12
Shoot	779	449, 330	368, 296, 105, 10	283, 244, 147, 59, 39, 7

The basic local alignment search from the National Center for Biotechnology Information (using the Blast tool of NCBI) performed for the stem (gb: KR706029) and shoot (gb: KR706030) of the fig *Phytophthora*, corroborated the results obtained in the enzymatic digestion, where the stem isolate presented a 99% identity match with the accessions KF559323.1, KC478663.1, JX996044.1, HQ643189.1andFJ801816.1of *P. cinnamomi*, while they obtained a 95% match with the accession AF266762.1 of *P. fragariae*, while the shot isolate presented an average 99% match with the accessions AF266780.1, GU258862.1, LM650992.1, KJ755111.1 and AB769175.1 of *P. palvimora*.

These results were also confirmed through the phylogenetic reconstruction using the Maximum Likelihood (ML) method performed to analyze the evolutionary relationships between the sequences of

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interest with the *Phytophthora*accessions reported in GeneBank (Figure-5).

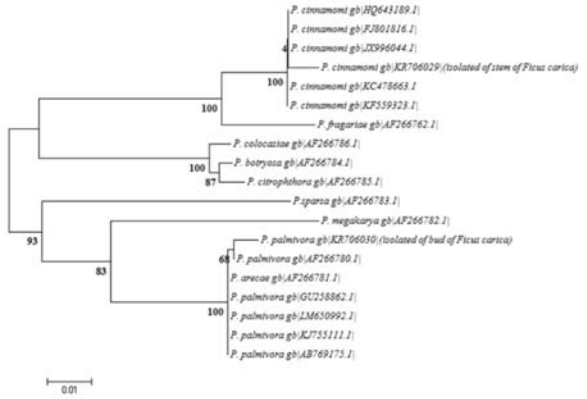

Figure-5. Reconstruction of phylogeny based on the *Phytophthora* rRNA fragment ITS1-5.8S-ITS2 using the Maximum Likelihood (ML) method with a 500 bootstrap.

DISCUSSIONS

Collecting symptomatic material, phytopathogen isolation and purification

According to Echemendía (2005), the isolation of *Phytophthora* species from infected plant tissue, is considered complicated due to the inteference that can occur from other pathogens with a more accelerated growth, as it is the case of species from the *Pythium*genus. Therefore, in this study, the V8 agar with Benomil culture medium was used, since authors such as Lopéz and Tomás (1999) have observed that the growth rate from *Phytophthora* species increases in cuture media supplemented with V8; enhancing the growth of this fungi and reducing the risk of contamination.

In general, the growth of *Phytophthora* in a Petri dish is observed through the development of white colonies(color independent from the culture medium used), which in most cases present a regular edges, except when using PDA medium (Potato Dextrose Agar), where it presents a reduced growth and the mycellum attached to the susbtrate (Cotilla*et al*., 2007). On the other hand, reports performed by González *et al*. (2014), establish that the colonies of *Phytophthora* spp. in media containing V8 juice are characterized by a cottonlike consistency, related to the high level of ramifications and mycellium density. The aspects described before were observed in the results obtained in this study when performing the Petri dish isolation assays, where aerial mycelium with starred and radiated growth was observed (Figure-1).

The addition of benomil in the culture medium provided a more selective substrate for Oomycota growthsince most of the fungi contain ergosterol as a main component of the cell membrane, which presents an important structural function besides being the vitamin D precursor in fungi (Laijnhouwers& Pierre, 2003). Within this context, studies performed by Machado et *al*. (2006) have determined that the ergosterol productions in fungi are reduced in the presence of benomil.

Finally, the exposure of the *Phytophthora* isolates to sterile rain water was also important for the isolation and purification process, since as proposed by Ristaino and Gumperts (2000), the presence of humidity is essential for the development of sporangia, since the zoospores that are formed within this structure require wáter for their development; an aspect which contributed to increasing the concentration of the microorganism of interest.

Morphological identification of the pathogen through Optical Microscopy (OM) and Scanning Electron Microscopy (SEM)

The morphological analysis for the generation of taxonomical classifications can be considered vague, due to the phenological plasticity and the genetic variability of the elements used; leading to incorrect classifications since many morphological key traits are only effective in a particular stage of development (Jarman & Elliott, 2000; Luque& Díez 2002). Nevertheless, despite this variable, it was possible to find reports of *Phytophthora* species presenting similar characteristics to those from the fig stem and shoot isolates (Figures 2 and 3). Considering this, Mansilla *et al*. (1993) determined that the presence of non-papilated sporangia with a slight apical thickening, ovoid-shaped and with sizes ranging between the 18 to 43µm and the presence of abundant chlamydospores in V8 culture medium are characteristic indicators for*P. cinnamoni*. On the other hand, Páez *et al*. (1993) have indicated that *P. palmivora*in V8 medium presentaerial mycellium, with radial growth where papilated and ellipsoid shaped sporangia can be observed and where size is not a taxonomic descriptor, due to the fact that it has not been possible to establish a relationship between the size ranges and the shape. The aspects mentioned before, present certain similarities with the description of the microorganism isolated from the fig shoot, since even though it was not possible to observe mature sporangia in most cases, it was possible to identify differences in the sizes and shapes of these structures.

Total DNA extraction, PCR-RFLP and bioinformatic analysis

The total DNA extraction was performed from the mycellium, in order to obtain a higher quantity of genetic material, since various authors mention the difficulties regarding the spore DNA extraction (Cassago *et al*., 2002; Lurá *et al*.) Through the use of liquid nitrogen and the maceration along with the lysis buffer, it was posible to achieve an efficient disruption, which allowed tosolubilize the nucleic acids and also inactivate the enzymes which may degrade DNA (Ronsted *et al*., 2007). An average purity value of 1.77 was obtained, which favoured the posterior amplification and sequencing of the bands of interest (Glasel, 1995). Additionally, the average 788bp amplified region of the ITS1, 5.8S and ITS2 of the fig isolates (Figure-4), was very similar to the one reported by Drenth *et al*., (2006),who obtained in average 808 bp

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using the same primers for the identification of *P. palmivora* and *P. cinnamomi*.

Table-2. Genetic distance matrix of the ML tree.

Accessions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. *P. fragariae* gb|AF266762.1|		0,007	0,007	0,007	0,007	0,007	0,008	0,012	0,012	0,012	0,015	0,014	0,014	0,014	0,014	0,014	0,014	0,014	0,014
2. *P. cinnamomi* gb|KF559323.1|			0,000	0,000	0,000	0,000	0,003	0,012	0,012	0,012	0,014	0,013	0,013	0,013	0,012	0,012	0,012	0,012	0,012
3. *P. cinnamomi* gb|KC478663.1				0,000	0,000	0,000	0,003	0,012	0,012	0,012	0,014	0,013	0,013	0,013	0,012	0,012	0,012	0,012	0,012
4. *P. cinnamomi* gb|JX996044.1|					0,000	0,000	0,003	0,012	0,012	0,012	0,014	0,013	0,013	0,013	0,012	0,012	0,012	0,012	0,012
5. *P. cinnamomi* gb|HQ643189.1|						0,000	0,003	0,012	0,012	0,012	0,014	0,013	0,013	0,013	0,012	0,012	0,012	0,012	0,012
6. *P. cinnamomi* gb|FJ801816.1|							0,003	0,012	0,012	0,012	0,014	0,013	0,013	0,013	0,012	0,012	0,012	0,012	0,012
7. *P. cinnamomi* gb|KR706029|								0,012	0,012	0,013	0,015	0,014	0,014	0,013	0,013	0,013	0,013	0,013	0,013
8. *P. colocasiae* gb|AF266786.1|									0,004	0,004	0,013	0,012	0,012	0,011	0,011	0,011	0,011	0,011	0,011
9. *P. botryosa* gb|AF266784.1|										0,003	0,013	0,012	0,012	0,012	0,011	0,011	0,011	0,011	0,011
10. *P.citrophthora*gb|AF266785.1|											0,013	0,013	0,013	0,012	0,012	0,012	0,012	0,012	0,012
11. *P.sparsa* gb|AF266783.1|												0,013	0,012	0,012	0,012	0,012	0,012	0,012	0,012
12. *P.megakarya* gb|AF266782.1|													0,010	0,010	0,010	0,010	0,010	0,010	0,010
13. *P.palmivora* gb|KR706030|														0,003	0,003	0,003	0,003	0,003	0,003
14. *P.palmivora* gb|AF266780.1|															0,001	0,001	0,001	0,001	0,001
15. *P.arecae* gb|AF266781.1|																0,000	0,000	0,000	0,000
16. *P. palmivora* gb|GU258862.1|																	0,000	0,000	0,000
17. *P. palmivora* gb|LM650992.1|																		0,000	0,000
18. *P. palmivora* gb|KJ755111.1|																			0,000
19. *P.palmivora* gb|AB769175.1|																			

*Accession 7 and 13 are the stem and shoot isolates from the symptomatic fig tree samples.

According to Cooke*et al*. (2000), the intraspecific variation of the rDNA of individuals from the same species is low and stable but not absent, which is a trait that has contributed to the identification of *Phytophthora* species based in the study of the ITS region, where Cooke*et al*. (2005) and also Drenth *et al*. (2006), have standardize genetic fingerprint patterms using the ITS1-5,8S-ITS2 region. However, it is important to consider that the patterns of PCR-RFLP may not always be effective for the generation of an organism specific genetic fingerprint, due to the fact that the restriction enzyme used to cut some or all the PCR product amplicons in a specific site may fail (Drenth *et al*., 2006). This can occur when the PCR amplicons generated through rRNA are copies of the genomic DNA of sequences that present tandem repetitions (polymorphic), which hinder the possibility to differentiate between species (Kroon*et al*., 2004). Therefore, this study was performed including a phylogenetic analysis which complemented the PCR-RFLP pattern (Table-1), since through the use of the DNA of the microorganism isolated from the stem it was possible to achieve restriction patterns very similar to those reported by Drenth *et al*. (2006) for*P. Cinnamoni* and *P. fragaria;* while for the microorganism isolated from the fig shoot, the restriction pattern obtained was similar to the one for *P. palmivora*. These results are similar to those described by Kroon *et al*. (2004), where it is established that, in most cases, these tandem repetitions are generally uniform; hence, PCR-RFLP patterns can be used as a preliminary distinction factor, previous to sequencing and phylogenetic analysis.

The use of Blast in the amplified ribosomal RNA region of the studied isolates showed up to 99% homologies with species identified in the database. On the other hand, the phylogenetic analysis of the strains presented a similar behavior to the results obtained from the Blast search (Figure-5), where the microorganism isolated from the fig stem was grouped in the same accession node as *P. cinnamoni*, while the isolate derived from the shoot was found more closely related to the accessions for *P. palmivora*. According to Mendoza (2012),the more common ancestors being shared by two taxons, the more related they are to one another. This was evidenced when the genetic distance matrix for the *Phytophthora* isolated was analyzed (Table 2), where the divergence between the *P. cinnamoni* taxons in regards to the microorganism isolated from the stem was 0.007, while the microorganism isolated from the fig shoot presented an average divergence of 0.0068 when compared to the taxons corresponding to *P. palmivora.*

These results match those reported by Sanz *et al*. (2004)who indicate that the coding sequence of the ribosomal RNA contribute to the genotype segregation, product of the polymorphisms present in each taxon; allowing to explore the genetic diversity and the establishment of relations within genotypes. Within this context, it is possible to confirm the statement made by Beiggi and Piercey (2007) as well as Besse (2014), who reported the efficiency of the sequence ITS1-5,8S-ITS2 for the development of phylogenetic and evolutionary relationships, due to the highly conserved 5.8S ARNr region, next to the two highly variable regions (ITS1 e ITS2), which present variation even within species (Deng*et al*., 2008).

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